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Toxicity of 2,3,5,6-tetrachlorophenol to willow trees (*Salix viminalis*)

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Abstract

Chlorinated phenols have been intensively investigated from an eco-toxicological point of view, however almost nothing is known about toxicity of tetrachlorophenol (TeCP) to higher terrestrial plants. This paper applied the willow tree acute toxicity test by Trapp et al. (2000) and studied the toxicity of 2,3,5,6-TeCP to willows (*S. viminalis*) at neutral and acidic conditions (roughly pH 7 and 4) with inhibition of transpiration as toxic endpoint. At neutral pH the EC_{50} was >10 mg/L while the EC_{50} at acidic conditions was 0.32±0.17 mg/L, clearly indicating that toxicity is exerted by the non-ionic chemical fraction. Standard tests running at neutral pH are therefore not capturing the full toxicity of weak acids and bases.

1 Introduction

Chlorinated phenols have been widely used as pesticides, disinfectants and wood preservation agents in the sawmill industry in both Europe and the United States of America (Kitunen et al., 1987; Rappe et al., 1978). The main components of the wood preservation agents used were tri-, tetra- and pentachlorophenol (Kitunen et al., 1987;

Kitunen et al., 1984). The high polychlorinated phenols are classified as very toxic to aquatic organisms and some of them are suspected carcinogens, thus constituting a potential risk to human and environmental health (ECHA, 2017). Recent studies indicate that some of the higher chlorinated phenols may generate multi resistant organisms in the environment (Muller, 2015). Due to their wide industrial applications, the toxicity of chlorinated phenols has been intensively investigated (Aruoja et al., 2011; Hulzebos et al., 1993; Sharma et al., 1997; Shigeoka et al., 1988). However, to the knowledge of the authors, the only existing phytotoxicity studies available for tetrachlorophenol (TeCP) on terrestrial plants, are with 2,3,4,5-TeCP on soybean (*Glycine max* L.) and barley (*Hordeum vulgare* L.) (Pfleeger et al., 1991) and a study of 2,3,4,6-TeCP on radish (*Raphanus sativus* L.) and Sudan grass (*Sorghum sudane*) (Sund and Nomura, 1963), neither of them consider the effects of pH. The chlorinated phenols are weak acids, i.e. non-ionic at low pH. It is well-established that the uptake of ionized compounds is lower than for their corresponding non-ionic fraction, resulting in higher toxicity of non-ionized electrolytes (Clausen and Trapp 2017; Rendal et al. 2011; Sijm et al. 2007; Trapp 2004; Saarikoski and Viluksela 1982). The pKa of TeCP varies between 5 and 7 depending on the isomer (Schultz 1987; Schwarzenbach and Westall 1985; Lepri et al. 1980), meaning that both the ionized and the neutral fractions occur under natural environmental conditions (pH 4-10) (Franco et al. 2010). This study aims to assess the phytotoxicity of 2,3,5,6-tetrachlorophenol (2,3,5,6-TeCP) to terrestrial plants at neutral (pH 7) and acidic (pH 4) conditions.

2 Materials and methods

2.1 Experimental setup

The willow tree acute toxicity test by Trapp et al. (2000) was used to derive the effective concentration exerting effects on 50% of the population (EC50) in a preliminary range

finding experiment for 2,3,5,6-TeCP at pH 7 and 4 (exact pH-values are provided in Table 2) with a subsequent refined test at pH 4. The nominal concentrations tested were 0, 0.1, 1 and 10 mg TeCP/L and 0, 0.2, 0.5 and 1 mg TeCP/L for the range finding tests and the refined test, respectively. Aqueous concentrations at test termination were not measured. The aqueous solubility of 2,3,5,6-TeCP is reportedly 0.1 g/L at 25°C (Ma et al., 1993) and 0.05 g/L at 20°C (Albanis et al., 1998), with an estimated vapour pressure of 0.02 Pa (HSDB database by Toxnet). Using these data to calculate a dimensionless partitioning coefficient between air and water gives $2.2*10^{-5}$, which corresponds well to the Henry's law constant of $3.5*10^{-2}$ $Pa*m^3*mol^{-1}$, estimated by a QSAR by Meylan and Howard (1991). Based on this, volatilization from water is expected to be negligible. 40 cm willow cuttings of *Salix viminalis* (*S. viminalis*) were pre-grown in a bucket of tap water at room temperature. When the cuttings sprouted and the leafy branches reached a length of approximately 30 cm the willows were transferred to 0.5 L Erlenmeyer flasks, wrapped in aluminum foil and filled with 400 mL modified ISO 8692 nutrient solution. All flasks were sealed by aluminum foil and parafilm to avoid direct evaporation. The two pH-levels used were controlled by changing the nitrogen source of the nutrient solution from nitrate to ammonium and omitting the sodium bicarbonate buffer, as proposed by Mikes and Trapp (2010). The nutritional composition of the nutrient solution is presented in Table 1. The trees were fixed above the water surface so that only roots were in contact with the test solution to avoid adsorption to the submerged stem. After three days of adaptation the nutrient solution was exchanged by 2,3,5,6-TeCP-spiked nutrient solution. The temperature and humidity were approximately 15±3 oC and 55±10%, respectively. Toxicity was measured, at approximately 0, 24, 48 and 72 h and 144 h for the preliminary tests and at 0, 24 and 48 for the refined test. Effects on transpiration occur fast (within two days) why the refined test was terminated after 48h. This also minimizes the risk of diseases and pests. Toxicity was measured as decrease in transpiration (weight loss of the plant-flask-system) of the individual trees compared to the control trees and expressed as normalized relative transpiration (NRT%):

$$NRT(C,t)\ (\%) = \frac{\frac{1}{n}\sum_{i=1}^{n} T_i(C,t)/T_i(C,0)}{\frac{1}{m}\sum_{j=1}^{m} T_j(0,t)/T_j(0,0)} \times 100 \qquad \text{(Trapp et al. 2000)}$$

where C is TeCP concentration in solution (mg/L), t is the time period (h), T is the absolute transpiration (g/h), and the indices, i and j, indicate the replicates of test plants and control plants, respectively (n = m = 5).

Table 1 Composition of the ISO 8692 nutrient solution.

Macronutrient	Conc. (mg/L)	Micronutrients	Conc. (µg/L)
$NaNO_3$*	240	$FeCl_3 \cdot 6H_2O$	100
NH_4Cl*	53	$Na_2EDTA \cdot 2H_2O$	200
$MgCl_2 \cdot 6H_2O$	12	H_3BO_3	185
$CaCl_2 \cdot 2H_2O$	18	$MnCl_2 \cdot 4H_2O$	415
$MgSO_4 \cdot 7H_2O$	15	$ZnCl_2$	3
KH_2PO_4	32	$CoCl_2 \cdot 6H_2O$	1.5
$NaHCO_3$*	150	$CuCl_2 \cdot 2H_2O$	0.01
		$Na_2MoO_4 \cdot 2H_2O$	7

EDTA: ethylene-diamine-tetra- acetic acid
* NH_4Cl substituted $NaNO_3$ and the buffer $NaHCO_3$ when testing at pH 4.
Table is modified from Mikes and Trapp (2010)

2.2 Statistics and statistical software

One-way ANOVAs were performed to test for statistical differences of the mean of the treatment groups, with subsequent Bonferroni post hoc test. For data with unequal variances a Kruskal-Wallis test was performed followed by Dunn's post hoc test. The ANOVAs were performed in GraphPad Prism with an error probability, α, of 0.05. For generation of the dose-response curve the method described by Christensen et al. (2009), assuming a logarithmic-normal distribution of data and using a logit transformation, was applied.

3 Results and discussion

The toxicity of 2,3,5,6-TeCP to willow trees at pH 7 and 4 was tested. Figure 1 presents the NRT(%) and the average transpiration (g/d) for the range finding test at pH 7 (Figure 1A,B), the corresponding test at pH 4 (Figure 1C,D) and the final experiment at pH 4 with refined concentration range (Figure 1E,F). At neutral pH the effect of 2,3,5,6-TeCP on transpiration of the willows is weak and statistically insignificant ($P > 0.05$) even at the highest tested concentration (10 mg/L). In comparison, the toxicity exerted at acidic conditions is much higher. Here the two highest concentrations (10 mg/L and 1 mg/L) reduced the NRT(%) and the actual transpiration rates more than 75% within 48h (Figure 1C). In both cases the inhibition was statistically significant ($P < 0.05$). In the preliminary range finding test, at nominal pH of 4, concentrations of 0.1 mg/L and lower did not exert statistically significant toxicity to the willows ($P > 0.05$). These findings are also supported by the growth of the willows (Table 2), which indicates that the trees exposed to 1 and 10 mg/L at low pH were wilting (negative growth). The refined toxicity study at pH 4 indicates a clear dose response relationship as evident from Figure 1E-F and Figure 2. In the refined study, the highest tested concentration of 1 mg/L reduced the NRT(%) and the actual transpiration rate by more than 80% within 48h of exposure which was statistically significant ($P < 0.05$).

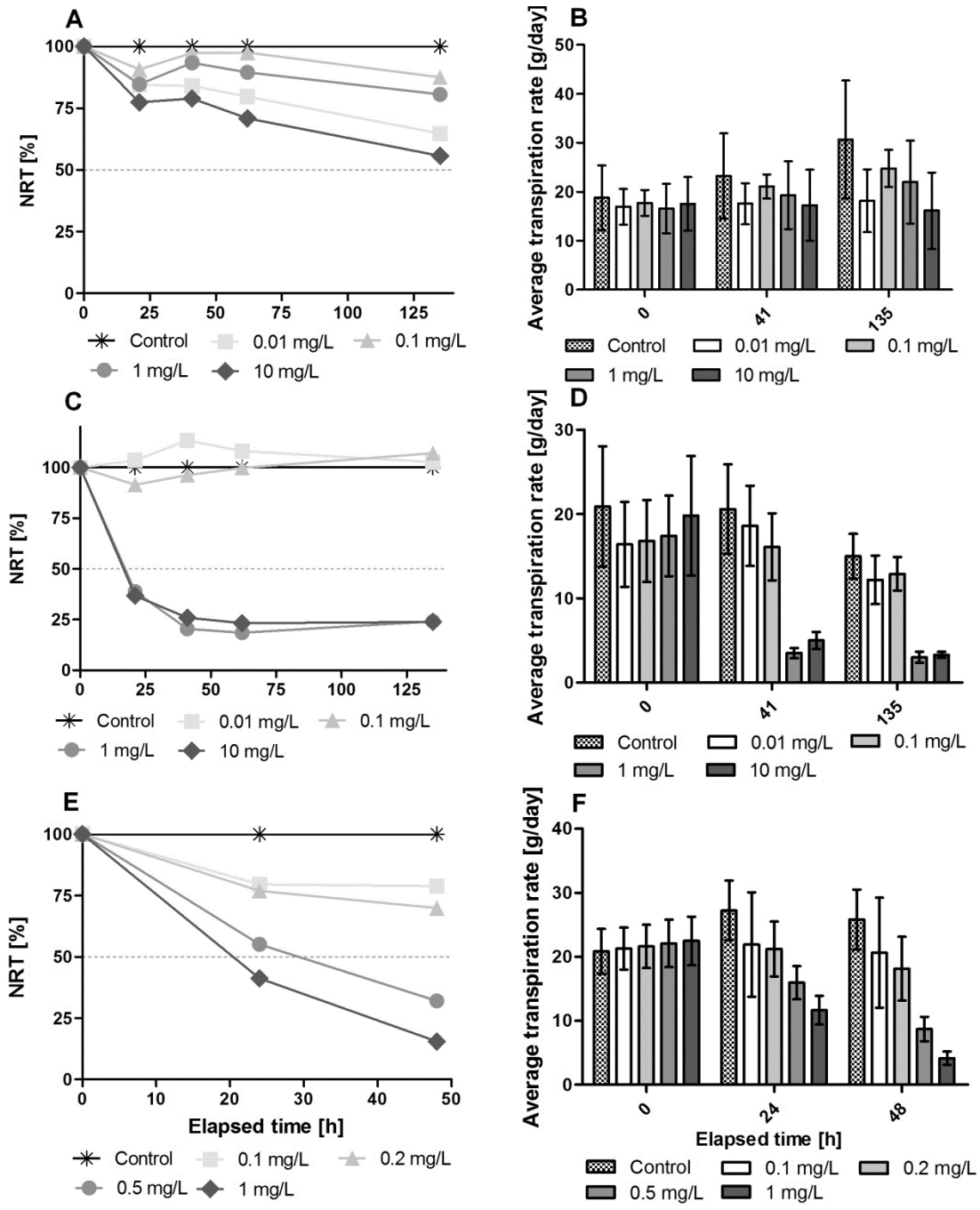

Figure 1. Visual representations of the NRT (%) (A,C,E) and average transpiration rate (g/day) (B,D,F) for the range finding experiment at high pH (A and B), the range finding experiment at low pH (C and D) and the final experiment with low pH (E and F). The error bars in the column plots denote standard deviations among replicates.

Table 2 pH and weight change of the treatments for range finding tests and the refined test. The bracketed numbers denotes the standard errors.

	pH, initial	pH, final	Weight change (%)
Range-finding, neutral pH			
Controls	8.1 (0.1)	6.7 (0.1)	7.4 (4.4)
0.01 mg/L	8.0 (0.1)	6.7 (0.1)	5.4 (0.8)
0.1 mg/L	8.2 (0.1)	6.7 (0.1)	4.8 (6.7)
1 mg/L	8.2 (0.02)	6.9 (0.1)	6.6 (1.0)
10 mg/L	8.0 (0.1)	7.0 (0.1)	0.8 (1.8)
Range-finding, low pH			
Controls	5.1 (0.2)	3.7 (0.1)	0.9 (3.1)
0.01 mg/L	5.7 (0.3)	3.7 (0.1)	2.1 (1.4)
0.1 mg/L	5.8 (0.4)	3.6 (0.1)	2.3 (1.5)
1 mg/L	5.5 (0.4)	4.7 (0.2)	-10.3 (1.4)
10 mg/L	5.2 (0.3)	5.1 (0.1)	-12.7 (3.0)
Refined, low pH			
Controls	5.1 (0.1)	3.5 (0.03)	nm
0.1 mg/L	5.0 (0.1)	3.5 (0.04)	nm
0.2 mg/L	5.1 (0.1)	3.5 (0.04)	nm
0.5 mg/L	5.1 (0.03)	3.5 (0.03)	nm
1 mg/L	5.0 (0.04)	4.7 (0.2)	nm

nm: not measured

Table 2 shows the measured initial and final pH of the test solutions of the three experimental setups (the range finding tests at neutral and acidic conditions and the refined test at acidic conditions). With nitrate as nitrogen source and sodium carbonate as buffer, initial pH is 8-8.2, and the final pH is 6.8-7, i.e. the solution is neutral. With ammonium as nitrogen source and unbuffered, the initial pH varies from 5-5.5. The final pH depends on the health of the trees and thus also on the dosage of TeCP. The limited transpiration at high test concentrations results in reduced uptake of nitrogen and, in the case of ammonium as nitrogen source, less protons excreted by the plants. Final pH ranges from 3.5-4.7 with the highest pH observed for the high exposure groups. The pKa of 2,3,5,6-TeCP is reported by Schultz (1987), Lepri et al. (1980) and Doedens (1965) to 5.14, 5.44 and 5.48, respectively, resulting in approximately 97-99% ionization at pH 7 and approximately 3.2-6.8% ionization at pH 4, when calculated by the Henderson-Hasselbach equation. This is also confirmed by our results (Figure 1 and Table 2). The

EC50 is >10 mg/L at pH 7 and 0.32±0.17 mg/L at pH 4 (deviation is 95% confidence interval, Figure 2), clearly showing the increased uptake and thus toxicity of the non-ionic species. In comparison, the EC50-values obtained by Pfleeger et al. (1991) for 2,3,4,5-TeCP on soybean and barley were 1±0.2 mg/L and 7.6±1.3 mg/L, respectively, (deviation reported are standard errors) in a hydroponic experiment. For 2,3,4,6-TeCP Sund and Nomura (1963) reports EC50-values of 16 mg/L, and 67 mg/L for radish and sudan grass in a seed germination test on filter paper (no confidence intervals reported). The values are approximately one to two orders of magnitude higher than the EC50-value reported in this study for the non-ionized compound and is likely to be in the similar range for the ionized fraction. Neither Pfleeger et al. (1991) nor Sund and Nomura (1963) reported the corresponding pH values. Most likely, their results are valid for neutral pH.

Environmental concentrations of TeCP and other chlorophenols are in general low (in the µg/kg dry weight range) (Czaplicka, 2004) but in the proximity of industrial sites, e.g. wood preservation facilities, high concentrations have been observed (in the mg/kg dry weight range) (Knuutinen et al., 1990). Environmental concentration of TeCP may thus, at some industrial sites, be problematic from a phytotoxicity perspective at acidic conditions.

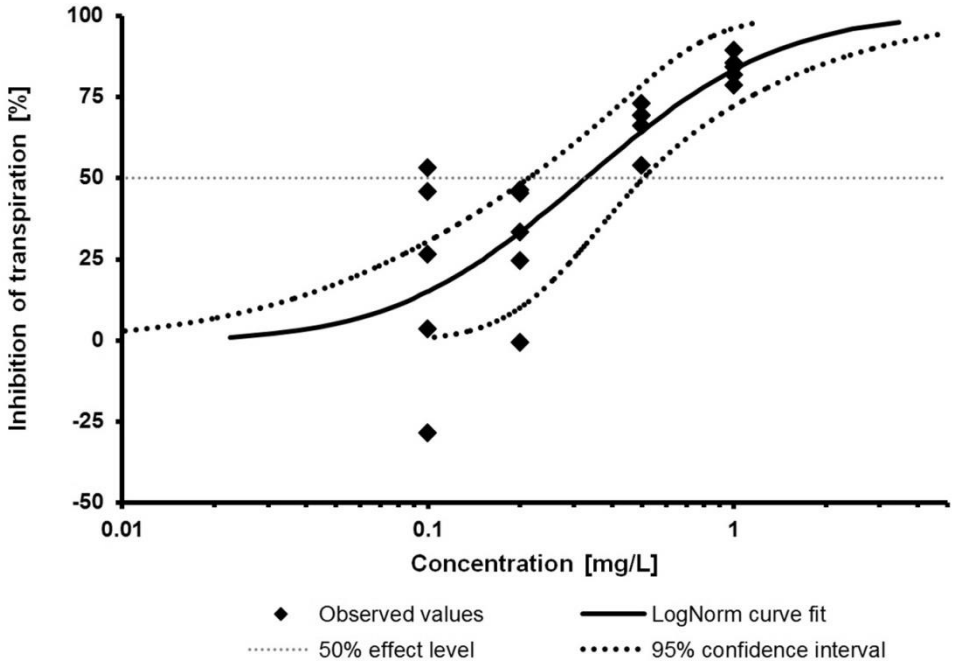

Figure 2. LogNorm fitted dose-response curve (bold black line) used to derive the EC_{50}-value for the low pH experiment with refined exposure concentrations with corresponding 95% confidence intervals (dotted lines).

As argued by e.g. Rendal et al. (2012), it has to be stressed that toxicity tests must be carried out at various pH regimes when assessing the risks of ionizing compounds. The necessity of such precaution is acknowledged in the REACH guidelines, stating that "it may be necessary to determine the toxicity of both anionic and cationic species" for compounds ionizing "to a significant extent" within pH 4-9 (ECHA 2016). Such precautions are not mentioned in the actual REACH regulation (ECHA 2006).

For the sake of plant health, standardized toxicity tests for terrestrial plants advice to use a pH between 6 and 7.5 in soil tests (ISO 2005; OECD 2006a; OECD 2006b; ASTM 2014). In this study the willow biomass increased by 0.9 % at low pH, while increasing by 7.4 % at neutral pH (Table 2), indicating stress in the low pH regime. It must be noted that such stress can potentially cause elevated sensitivity of the willows towards the test substance, in which case the actual toxicity might be overestimated. On the other hand, the lower transpiration rate at low pH lead to lower exposure, which will affect the result

in the opposite direction, i.e. the toxicity may be underestimated. Work remains in pursuit of standardized terrestrial plant tests suitable for low and high pH regimes.

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